REMARKS

Claims 31-39 are pending. Non-elected claims 31-36 are canceled without prejudice. Claims 37-39 are rejected.

Regarding the Examiner's non-consideration of reference J.R. (Sunthankar) in the February 11, 2004 IDS, Applicants submit the reference in a Supplementary Information Disclosure Statement and acknowledge the Examiner's agreement to consider it.

The Specification is amended to use the conventional nomenclature ST for all disclosure of ST receptor binding compounds. ST is the same bacterial (*E. coli*) peptide toxin disclosed in Applicants' originally filed specification. The amendment simply uses the more conventional designation, omitting qualifiers such as bacterial source and temperature characterizations, which are known (see, e.g., the 1986 J. Biol. Chem. reference attached in support), and corrects typographical errors.

CLAIM REJECTIONS UNDER 35 U.S.C. § 112

Claims 37-39 are rejected under 35 U.S.C. § 112 ¶2 as indefinite.

Responding to the Examiner's ¶6, claim 37 is amended to recite compounds encompassed by Type 1 and Type 2 agents, and the chemical nature and structure, namely E - L - DYE - X - N_3.

Responding to the Examiner's ¶7, Applicants assert that one skilled in the art would know or could determine without undue experimentation what power and fluence would be sufficient for necrosis or apoptosis (as a non-limiting example, determining extent of apoptosis using either Annexin V or caspase assays, determining extent of necrosis using trypan blue dye exclusion).

Responding to the Examiner's ¶8, claim 38 depends from and thus further limits claim 37. As known to one skilled in the art, N_3 is the chemical structure of azide. Type 1 and Type 2 agents, and their chemical nature and structure, are clearly defined, at least by the following disclosure.

The compound is E - L - DYE - X - N_3 where substituents for each of E, L, DYE, and X are provided (p. 6 line 5 to p. 8 line 1).

The Type 1 component is provided by N_3 (azide). N_3 produces nitrene upon photoactivation (p. 11 line 14, also Sunthankar et al. Reactive disperse dyes. 1. Reactivity involving nitrene intermediate from azido group. Indian Journal of Chemistry (1973) 11, 503, which Applicants have incorporated by reference (p. 13 lines 12-16). The inventors have provided data (see attached Declaration under 37 C.F.R. § 1.132) showing that the azide moiety, upon exposure to light, causes cell death and thus demonstrating that N_3 is a Type 1 agent that acts via a nitrene intermediate.

The Type 2 component is provided by DYE. DYE is disclosed as an aromatic chromophore that undergoes photosensitization and produces singlet oxygen for PDT (p. 11 lines 14-16, FIG. 1 "Photosensitizer (DYE)"; PDT agents are Type 2 agents ("Most of the currently known photosensitizers are commonly referred to as photodynamic therapy (PDT) agents and

operate via the Type 2 mechanism" (p. 4 lines 22-23); "A homogeneous photosensitizing mixture consisting of two or more Type 2 (PDT) agents is prepared", p. 8 lines 14-16). DYE may be an aromatic or a heteroaromatic radical derived from cyanines, indocyanines, phthalocyanines, rhodamines, phenothiazines, fluoresceins, porphyrins, benzoporphyrins, or corrins (p. 10 lines 13-16).

Responding to the Examiner's ¶9, claim 39 is amended to clarify that accumulation occurring before exposure to light.

Claims 37-39 are rejected under 35 U.S.C. § 112 ¶1 as not enabled for all azides. The amended claims recite E - L - DYE - X - N_3, thus overcoming this rejection.

Because each of the above rejections under 35 U.S.C. §112 are now overcome, Applicant respectfully requests its withdrawal.

CONCLUSION

Applicants authorize a credit card charge for a one-month extension of time (see electronic fee sheet) no other fees are believed due. If any fees are deemed necessary, the Examiner has authorization to charge them to Deposit Account No. 23-3000.

The Examiner is invited to contact Applicant's undersigned representative with any questions.

Respectfully submitted,
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Characterization of the Receptor for Heat-stable Enterotoxin from *Escherichia coli* in Rat Intestine*

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The receptor for the heat-stable enterotoxin (ST) from *Escherichia coli* was solubilized with Lubrol-PX from rat intestinal brush-border membranes and characterized. The binding kinetics and analog specificity of the solubilized receptor were virtually identical to those obtained with the membrane-bound receptor. Furthermore, the regulation of the receptor's affinity by cations was also maintained after solubilization, indicating a conservation of the toxin-binding site after removal of the receptor from its membrane environment.

Gel filtration and sucrose density gradient sedimentation studies gave a Stokes radius of 5.5 nm and a sedimentation coefficient of 7.0 S for the solubilized receptor. The isoelectric point of the receptor was determined as 5.5 using Sephadex isoelectric focusing electrophoresis. In all of these separation techniques, the ST receptor showed a single peak of activity that was clearly separated from that of guanylate cyclase. When ^{125}I-ST was cross-linked to brush-border membranes with disuccinimidyl suberate, the affinity-labeled receptor solubilized with 0.1% Lubrol-PX eluted at a similar position as the native receptor on gel filtration chromatography. Analysis of the affinity-labeled receptor by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the presence of reducing agent and by autoradiography revealed the presence of three specifically labeled polypeptides with apparent molecular weights of 80,000, 68,000, and 60,000.

These results suggest that the ST receptor is solubilized by Lubrol-PX in an active form with preservation of its regulation by cations. Also, the ST receptor is separable from particulate guanylate cyclase indicating that the receptor is coupled to the activation of guanylate cyclase by an as yet undefined mechanism. Three subunit peptides may constitute a binding region of the receptor.

Heat-stable enterotoxins (ST[1]) produced by pathogenic strains of *Escherichia coli* are low molecular weight peptides that cause watery diarrhea by stimulating intestinal secretion (1–5). Several studies have provided evidence in favor of the concept that binding of ST to its specific receptor leads to the activation of particulate guanylate cyclase that initiates a cascade of reactions culminating in changes in ion and fluid transport in the brush-border membrane of intestinal epithelial cells (6–9). Although most mammalian tissues contain both soluble and particulate forms of guanylate cyclase, the enzyme in intestinal mucosa is predominantly particulate (10, 11). ST activates only the particulate form of the enzyme in intestinal mucosa (6–9). Recently, a high-affinity receptor for ST associated with rat intestinal epithelial cells and brush-border membranes has been detected (12–14) and solubilized (15). However, little is known about the coupling mechanism between the ST receptor and particulate guanylate cyclase. It remains to be determined whether the ST receptor and particulate guanylate cyclase are separate macromolecules or constitute a single protein that has an ST-binding site and guanylate cyclase activity. de Jonge (16) proposed that these activities reside in a single protein.

In this report, we describe the solubilization of the ST receptor with the nonionic detergent Lubrol-PX. Using several techniques, solubilized ST receptor and particulate guanylate cyclase can be separated indicating that they are different macromolecules. The structure of the ST receptor was also studied using an affinity cross-linking method. Some of these observations have been presented in abstract form (17).

EXPERIMENTAL PROCEDURES

Materials—Native ST purified from *E. coli* strain 431 was generously provided by Dr. D. C. Robertson, University of Kansas, Lawrence (13–15, 18). Synthetic analogs of ST used in this study included a 19-amino acid analog, a 14-amino acid analog (residues 6–19) and a 6-amino acid peptide (residues 10–15). The structure and synthesis of these peptides were described previously (19). Synthetic conotoxin GI was purchased from Peninsula Laboratories. Native ST or a synthetic 19-amino acid analog was radiolabeled with ^{125}I by the lactoperoxidase method ((13) Enzymobeads, Bio-Rad) to a specific activity of 300–700 Ci/mmol. Iodinated native toxin and synthetic 19-amino acid analog gave similar binding kinetics and were used interchangeably in these experiments. Disuccinimidyl suberate was purchased from Pierce Chemical Co., and bacitracin was obtained from Sigma. All other reagents were of analytical reagent grade and obtained as described previously (6, 8, 9, 20).

Membrane Preparation and Solubilization Procedure—Brush-border membranes were isolated from the small intestines of male Sprague-Dawley rats (weight 200–250 g) by a modification of the procedure described by Hauser *et al.* (21). Briefly, small intestine (jejunum and upper ileum) was removed and rinsed with ice-cold 50 mM Tris-HCl buffer, pH 7.6, containing 1 mM EDTA, 1 mM dithiothreitol, 0.25 M sucrose and 0.1 mM phenylmethanesulfonyl fluoride (buffer A). Mucosal scrapings were obtained with a glass slide. Mucosal tissue from four rats was homogenized with a Polytron homogenizer (Brinkmann) in 80 ml of ice-cold buffer A. The homogenate

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[1] The abbreviations used are: ST, heat-stable enterotoxin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate.

was diluted 6-fold with ice-cold distilled water and adjusted to 10 mM Mg^{2+} by the addition of solid $MgCl_2$. After incubation at 4 °C for 15 min with mixing, the diluted extract was centrifuged at 1,000 × *g* for 20 min at 4 °C. The pellet was discarded, and the supernatant fraction was centrifuged at 100,000 × *g* for 60 min. The supernatant fraction was discarded. The pellet was resuspended in ice-cold buffer A and was washed twice with buffer A by repeating the resuspension and centrifugation. The final suspension was used fresh or stored at −80 °C until used.

Preparations of brush-border membranes were homogenized with a glass-Teflon homogenizer in 50 mM Tris-HCl buffer, pH 7.6, containing 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethanesulfonyl fluoride, and 0.1% (w/v) Lubrol-PX (buffer B) at a protein concentration of 1 mg/ml. The homogenate was kept on ice for 2 h prior to centrifugation at 200,000 × *g* for 60 min. The supernatant fraction containing approximately 0.5–0.4 mg of protein/ml was used as a solubilized preparation.

Receptor-binding Assay—Reaction mixtures contained 20 μl of sample (membrane or solubilized preparations), 50 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, 150 mM NaCl, 0.67 mM cystamine, 0.1% (w/v) bacitracin, ^{125}I-ST, and the competing ligand, when used, in a final volume of 60 μl. Sodium chloride was omitted or substituted by other cations in some experiments. Cystamine was included in these reactions because samples contained dithiothreitol which inhibits the binding of ST to its receptor. Cystamine was used as a sink for the dithiothreitol and has no effect on receptor binding of toxin (data not shown). ^{125}I-ST and the competing ligand, when used, were combined in the assay tube followed by addition of samples to initiate the binding reaction. Nonspecific binding was determined using 1 μM nonradioactive 14-amino acid analog of ST. Reactions were allowed to proceed for 15 min at 37 °C to equilibrium. Bound ^{125}I-ST was separated from the free ligand by immediate filtration through polyethyleneimine-treated (22) Whatman GF/B or GF/C filters under vacuum. The reaction tube and filters were washed three times with 5 ml of ice-cold 20 mM phosphate-buffered saline, pH 7.0. Radioactivity on the filter was determined by counting in a Beckman Gamma 4000. The recovery of the solubilized receptor to the polyethyleneimine-treated filters in the binding assay was 95–105% when that of the gel filtration assay was assumed 100%. In some experiments, a cell harvester (M-24, Brandel, Gaithersburg, MD) was adapted for use in binding studies, and similar results were obtained.

Guanylate Cyclase Assay—Guanylate cyclase activity was assayed as described previously (6, 9, 20). Reaction mixtures contained 50 mM theophylline, 50 mM Tris-HCl, pH 7.6, 0.1% (w/v) bovine serum albumin, and a GTP-regenerating system consisting of 15 mM creatine phosphate and 20 μg (135 units/mg) of creatine phosphokinase. Assays (100 μl) were initiated by the addition of substrate (1 mM GTP and 4 mM $MnCl_2$), incubated for 10 min at 37 °C, and terminated by the addition of 50 mM sodium acetate, pH 4.0, followed by immersion in boiling water for 3 min. Generated cyclic GMP was quantified by radioimmunoassay (23) as described previously (8, 9, 20).

Gel Filtration—Gel filtration chromatography of the solubilized ST receptor was accomplished in two ways. The solubilized preparation (100 μl) or standard marker proteins (ferritin, catalase, lactate dehydrogenase, and bovine serum albumin) in a total volume of 100 μl were applied to a Spherogel-TSK G3000 SW column (7.5 × 300 mm; Beckman) that had been previously equilibrated and was then eluted with buffer B. The column was eluted with buffer B at 0.2 ml/min, and 0.2-ml fractions were collected. Each fraction was assayed for ^{125}I-ST binding and guanylate cyclase activity as described above. The elution positions of marker proteins were monitored by the absorbance at 280 nm.

Alternatively, the solubilized preparation (60 μl) was incubated with 1 nM ^{125}I-ST in the absence or the presence of 1 μM nonradioactive 14-amino acid analog of ST (as described in the binding assay above) in a final volume of 150 μl. After incubation at 37 °C for 15 min, 100 μl of the solution containing ^{125}I-ST-receptor complex was applied to the Spherogel TSK G3000 SW column, which was previously equilibrated and was eluted with buffer B without dithiothreitol at a flow rate of 0.2 ml/min. Each 0.2-ml fraction was assayed for radioactivity to locate the ^{125}I-ST-receptor complex.

Sucrose Density Gradient Centrifugation—Linear gradients (33 ml) were prepared with 5–20% (w/v) sucrose in buffer B. The solubilized preparation (1 ml) or standard marker proteins (β-galactosidase, catalase, lactate dehydrogenase, malate dehydrogenase, and cytochrome c) in a total volume of 1 ml were applied to the top of each gradient. Centrifugation was carried out at 4 °C in the Beckman SW 27 rotor at 135,000 × *g* for 24 h. After centrifugation, a capillary was lowered to the bottom of the centrifuge tube, and fractions of 0.85 ml were collected. Each fraction was assayed for ^{125}I-ST binding and guanylate cyclase activity as described above. Marker enzymes were assayed as described by Hall *et al.* (24).

Isoelectric Focusing Electrophoresis—The solubilized preparations (1 ml) were applied to a plate of Sephadex IEF (10 cm × 23 cm × 2 mm, Pharmacia) swollen with 6.3% (w/v) Pharmalyte (pH 5–8, Pharmacia) and 0.1% (w/v) Lubrol-PX and subjected to electrophoresis (30 watts for 2 h) at 4 °C, using a cathode solution of 0.5 M NaOH and an anode solution of 0.1 M H_3PO_4. After focusing, the Sephadex gels were cut into sections 7.5 mm wide, and each sample was extracted with 2 ml of buffer B containing 20% (v/v) glycerol. Samples were assayed for ^{125}I-ST binding and guanylate cyclase activity as described above. A part of each gel sample was extracted with distilled water for measuring pH.

Cross-linking Procedure and Analysis of Affinity-labeled Protein—Purified rat intestinal brush-border membranes prepared as described above were washed three times with ice-cold 20 mM phosphate-buffered saline, pH 7.0, by repeating the dilution and centrifugation. The washed membranes (1–2 mg of protein/ml) in 20 mM phosphate-buffered saline, pH 7.0, containing 0.1% (w/v) bacitracin were incubated for 15 min at 37 °C with 4 nM ^{125}I-ST in the presence or absence of 1 μM nonradioactive 14-amino acid analog of ST. After incubation, disuccinimidyl suberate (in dimethyl sulfoxide) was added to a final concentration of 1 mM, and the cross-linking reaction was allowed to occur for 15 min at 25 °C. Reactions were terminated by the addition of 0.05 volume of 1 M Tris-HCl buffer, pH 7.6. Lubrol-PX and phenylmethanesulfonyl fluoride were added to aliquots of quenched cross-linking reaction mixtures to final concentrations of 0.1% (w/v) and 0.1 mM, respectively. The samples were centrifuged at 200,000 × *g* for 60 min at 4 °C. Supernatant fractions (100 μl) were applied to the Spherogel TSK G3000 SW column, and each 0.2-ml fraction was collected and assayed for radioactivity to locate the ^{125}I-ST-cross-linked protein. Furthermore, affinity-labeled membrane and Lubrol-PX-solubilized preparations were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of 0.75-mm slab gels containing 5–15% or 7.5% acrylamide by the method of Laemmli (25). Samples were boiled for 3 min in the buffer containing 1.2% sodium dodecyl sulfate and 350 mM 2-mercaptoethanol prior to application to the gel. After electrophoresis, the gels were stained, destained, and dried for autoradiography. Molecular weight standards were phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and α-lactalbumin.

Proteins were determined by a modification (26) of the procedure described by Lowry *et al.* (27).

RESULTS

Conditions for Solubilization—We evaluated a wide range of detergents for use in solubilizing the ST receptor and particulate guanylate cyclase. Lubrol-PX was superior in extracting ST receptor and guanylate cyclase from rat intestinal brush-border membranes when compared with sodium deoxycholate, sodium cholate, CHAPS, Triton X-100, or digitonin. When intestinal brush-border membranes were solubilized at a protein concentration of 1 mg/ml, sodium deoxycholate (5 mM) and sodium cholate (25 mM) solubilized 75 and 38% of the ST receptor, respectively. However, both of them inhibited the particulate guanylate cyclase activity to less than 10% of the Lubrol-PX-solubilized preparations. CHAPS (10 mM) and Triton X-100 (0.3%) solubilized 21 and 23% of the ST receptor, respectively. However, they solubilized less than 10% of the particulate guanylate cyclase from the original brush-border membrane. Digitonin (1%) solubilized 38% of the ST receptor and 22% of the particulate guanylate cyclase. (The values are the means of two independent experiments that were done with triplicate determinations.) On the other hand, Lubrol-PX (0.1%) solubilized 54 ± 8% of ST receptor and 42 ± 6% of the particulate guanylate cyclase. (The values are mean ± S.E. of five independent experiments.) Higher detergent concentrations did not significantly increase the total activity solubilized; however, lower specific activities were

found due to an increase in total protein extracted from membranes (data not shown).

Binding Characteristics of Solubilized Receptor—Specific binding of ^{125}I-ST to purified brush-border membrane preparations was measured in the presence of increasing concentrations of ^{125}I-ST. As shown in Fig. 1, Scatchard analysis gave a linear relationship indicating a single class of specific binding sites. The B_{max} and K_D values obtained by computer fitting of data to a Scatchard plot were 5.4 ± 0.6 pmol/mg protein and 1.4 ± 0.2 nM, respectively. ^{125}I-ST binding to solubilized preparations resembled very closely the binding to membranes. Scatchard analysis of binding of ^{125}I-ST to solubilized preparations also revealed a single class of binding sites (Fig. 1). The solubilized receptor had a B_{max} value of 6.2 ± 0.5 pmol/mg protein and K_D value of 1.4 ± 0.2 nM, respectively.

To determine whether ^{125}I-ST binding sites in the membrane and solubilized preparations represent biologically relevant ST receptors, we evaluated the effects of several ST analogs to compete for ^{125}I-ST binding. As shown in Fig. 2*A*, the 19-amino acid synthetic analog and 14-amino acid synthetic analog of ST, which retain enterotoxic activity (9, 19), inhibited ^{125}I-ST binding to brush-border membrane preparations at concentrations of 1–100 nM. Conotoxin GI shares a partial sequence homology with ST, representing a common antigenic determinant, but has no enterotoxic activity (19). Conotoxin GI did not inhibit ^{125}I-ST binding. Similarly, a 6-residue synthetic peptide encompassing the region common to ST and conotoxin GI, which also has no enterotoxic activity, did not inhibit ^{125}I-ST binding. Inhibition curves of ^{125}I-ST binding to solubilized preparations by these four synthetic peptides were identical to those obtained with membrane preparations (Fig. 2*B*).

Specific ^{125}I-ST binding to membrane and solubilized preparations from rat intestinal brush border was altered by the

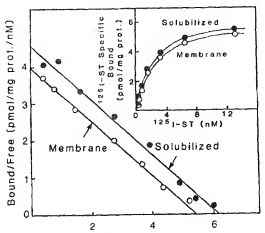

FIG. 1. **^{125}I-ST binding to membrane and solubilized preparations from rat intestinal brush border.** Scatchard plots and binding saturation curves (*inset*) for membrane (*open circles*) and solubilized (*closed circles*) preparations are shown. Aliquots (20 μl) of membrane or solubilized preparations containing 2 μg of protein were incubated with increasing concentrations of ^{125}I-ST for 15 min at 37 °C. Nonspecific binding was determined by parallel incubations in the presence of 1 μM 14-amino acid analog of ST. Binding data were plotted after correction for nonspecific binding. B_{max} and K_D values were 5.4 ± 0.6 pmol/mg protein and 1.4 ± 0.2 nM, respectively, for the membrane preparations and 6.2 ± 0.5 pmol/mg protein and 1.4 ± 0.2 nM, respectively, for the solubilized preparations. The values are mean ± S.E. of three experiments.

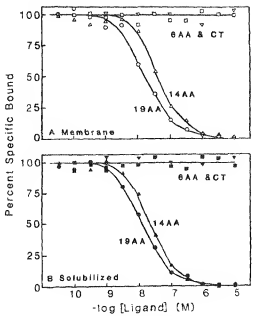

FIG. 2. **Inhibition of ^{125}I-ST binding to membrane (*A*) and solubilized (*B*) preparations from rat intestinal brush border.** Varying concentrations of peptides were incubated with 1 nM ^{125}I-ST and membrane (*open symbols*) or solubilized (*closed symbols*) preparations as described under "Experimental Procedures." Percentage values (*Percent Specific Bound*) were calculated by dividing the specific ^{125}I-ST binding at each point by maximum specific binding. Nonspecific binding was determined with 1 μM 14-amino acid analog of ST. The data presented are representative of three experiments with similar results. *19AA* (○, ●), 19-amino acid analog of ST; *14AA* (△, ▲), 14-amino acid analog of ST; *6AA* (□, ■), 6-amino acid residue of ST; *CT* (▽, ▼), conotoxin GI.

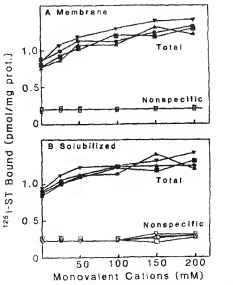

FIG. 3. **Effect of monovalent cations on ^{125}I-ST binding to membrane (*A*) and solubilized (*B*) preparations from rat intestinal brush border.** The figure displays total binding (*closed symbols*) and nonspecific binding (*open symbols*) of 1 nM ^{125}I-ST measured in the absence and in the presence of 1 μM 14-amino acid analog of ST. The results are the average of two separate experiments. ●, ○, Na; ▲, △, K; ■, □, Li; ▼, ▽, NH_4.

addition of various cations. Fig. 3 shows that inclusion of high concentrations of monovalent cations improved specific binding of ^{125}I-ST predominantly by increasing total binding without affecting nonspecific binding. All four (Li^+, Na^+, K^+, and NH_4^+) monoatomic cations when tested at 150 mM almost equally increased specific binding by about 80% in the membrane and by 60% in the solubilized preparations. As shown in Fig. 4, low concentration of divalent cations similarly enhanced specific binding of ^{125}I-ST. Addition of 8 mM Mn^{2+}, Mg^{2+}, and Ca^{2+} increased specific binding by 81, 67, and 37%, respectively, in the membrane preparations and 62, 29, and 17%, respectively, in the solubilized preparations. In contrast to monovalent cations, divalent cations increased nonspecific binding both in the membrane and solubilized preparations. In the absence of cation, B_{max} and K_D values obtained by fitting data to a Scatchard plot were 5.6 ± 0.5 pmol/mg protein and 2.9 ± 0.3 nM, respectively, for the membrane preparations and 6.4 ± 0.6 pmol/mg protein and 2.2 ± 0.2 nM, respectively, for the solubilized preparations (mean ± S.E. of three independent experiments). These results in the absence and presence of 150 mM NaCl (Fig. 1) indicate that these cations increased the affinity of the ST receptor for ^{125}I-ST without altering the receptor number. The divalent cations also affected only the K_D values, and the effects of divalent and monovalent cations on the specific binding were not additive (data not shown). In subsequent experiments binding was conducted in the presence of 150 mM NaCl, a concentration that was maximally effective. Divalent cations were not included because this increased nonspecific binding.

Characterization of the Solubilized ST Receptor—Some properties of the solubilized ST receptor and particulate guanylate cyclase were compared to determine if these activities reside on the same or different proteins.

To determine the Stokes radius of the ST receptor on gel filtration, the solubilized receptor was treated in two ways. In some experiments, the solubilized preparation was applied to a Spherogel-TSK G3000SW column, followed by elution in the presence of 0.1% Lubrol-PX. The collected fractions were assayed for ^{125}I-ST binding and guanylate cyclase activity (Fig. 5*A*). In other experiments, ^{125}I-ST was bound first to the solubilized receptor followed by application of the receptor-^{125}I-ST complex to the column (Fig. 5*B*). The time required for completion of chromatography was less than 1 h, during which there was minimal dissociation of ^{125}I-ST from the receptor. The peak of receptor-binding activity eluted at the same position relative to standard proteins in both types of experiments (Fig. 5). The Stokes radius was estimated at 5.5 ± 0.1 nm (mean ± S.E. of three experiments). In contrast, as shown in Fig. 5*A*, solubilized particulate guanylate cyclase activity eluted earlier than the ST receptor peak and migrated as a protein with a Stokes radius of 5.8 ± 0.1 nm (mean ± S.E. of three experiments).

Sedimentation coefficients of the ST receptor and particulate guanylate cyclase were estimated using a gradient of 5–20% sucrose (Fig. 6). Solubilized fractions were layered onto

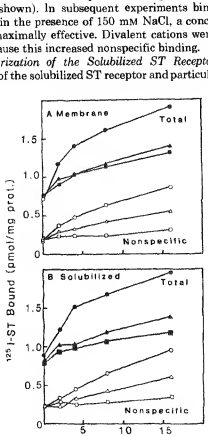

FIG. 4. **Effect of divalent cations on ^{125}I-ST binding to membrane (*A*) and solubilized (*B*) preparations from rat intestinal brush border.** The figure displays total binding (*closed symbols*) and nonspecific binding (*open symbols*) of 1 nM ^{125}I-ST measured in the absence and in the presence of 1 μM 14-amino acid analog of ST. The results are the average of two separate experiments performed in the absence of added monovalent cations. ●, ○, Mn; ▲, △, Ca; ■, □, Mg.

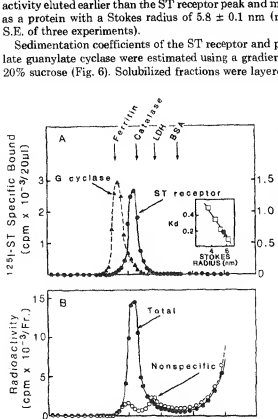

FIG. 5. **Gel filtration of solubilized ST receptor and particulate guanylate cyclase.** *A*, gel filtration of a solubilized preparation from rat intestinal brush border was followed by assay of fractions for specific ^{125}I-ST binding (^{125}*I-ST Specific Bound*, ●) and guanylate cyclase (▲) activities as described under "Experimental Procedures." The Stokes radii of ST receptor and guanylate cyclase were estimated at 5.5 ± 0.1 and 5.8 ± 0.1 nm, respectively. *B*, a solubilized fraction was incubated with 1 nM ^{125}I-ST in the presence (○, nonspecific) or absence (●, total) of 1 μM 14-amino acid analog of ST and then subjected to gel filtration. The Stokes radius of specifically labeled protein was estimated at 5.5 ± 0.1 nm. Markers were bovine serum albumin (*BSA*, 3.6 nm), lactate dehydrogenase (*LDH*, 4.5 nm), catalase (5.2 nm), and ferritin (6.1 nm). The values are mean ± S.E. of three experiments. The *inset* shows a standard curve of the distribution coefficient, K_d, *versus* Stokes radius of the calibrating proteins.

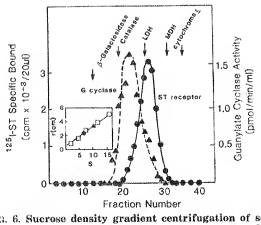

FIG. 6. **Sucrose density gradient centrifugation of solubilized ST receptor and particulate guanylate cyclase.** Sucrose density gradient centrifugation was carried out as described under "Experimental Procedures." Each fraction was assayed for specific ^{125}I-ST binding (*^{125}I-ST Specific Bound*, ●) and guanylate cyclase (▲) activities. The sedimentation coefficients of ST receptor and guanylate cyclase were calculated as 7.0 ± 0.4 and 10.0 ± 0.3 S, respectively. The markers were cytochrome *c* (1.9 S), malate dehydrogenase (*MDH*, 4.6 S), lactate dehydrogenase (*LDH*, 7.3 S), catalase (11.4 S), and β-galactosidase (16 S). The values are mean ± S.E. of three experiments. The *inset* shows a calibrating curve of distances (*r (cm)*) traveled by proteins *versus* S.

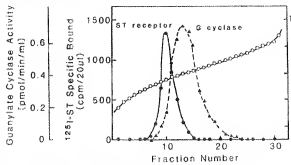

FIG. 7. **Isoelectric focusing of solubilized ST receptor and particulate guanylate cyclase.** Isoelectric focusing was carried out as described under "Experimental Procedures." Each fraction was assayed for ^{125}I-ST binding (●) and guanylate cyclase (▲) activities. The ST receptor and guanylate (*G*) cyclase had isoelectric points of 5.5 ± 0.2 and 5.9 ± 0.1, respectively. The values are mean ± S.E. of three experiments.

the gradients, and after centrifugation each fraction was assayed for ^{125}I-ST binding and guanylate cyclase activity. The peak of ^{125}I-ST binding with a sedimentation coefficient of 7.0 ± 0.4 S was clearly separated from the peak of guanylate cyclase with a coefficient of 10.0 ± 0.3 S (mean ± S.E. of three experiments) (Fig. 6).

The isoelectric points of the ST receptor and particulate guanylate cyclase were determined by isoelectric focusing of the Lubrol-PX-solubilized rat intestinal brush-border preparations as described above. The mobilities of the ST receptor and particulate guanylate cyclase are summarized in Fig. 7. The peak of ST receptor had an isoelectric point of 5.5 ± 0.2 and was separated from that of guanylate cyclase which had an isoelectric point of 5.9 ± 0.1 (mean ± S.E. of three experiments).

Affinity Cross-linking of ST Receptor—Analysis of affinity-labeled receptor was performed in two ways. Membranes containing cross-linked ^{125}I-ST were solubilized with 0.1% Lubrol-PX, and solubilized ^{125}I-ST-receptor complexes were applied to gel filtration as described above. The cross-linked ^{125}I-ST-receptor complex behaved similarly to its noncross-linked counterpart (see Fig. 5) and had a stokes radius of 5.5 ± 0.1 nm (mean ± S.E. of three experiments) (Fig. 8*A*). Furthermore, when cross-linked receptor was mixed with the noncross-linked control, a single peak was observed at the same elution position (data not shown).

With the receptor covalently linked to the labeled toxin, samples were also analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the presence of reducing agent and by autoradiography as described above. As shown in Fig. 8*B*, the pattern of labeled polypeptides obtained with either cross-linked membranes or cross-linked Lubrol-PX-solubilized preparations were identical. Three specifically labeled protein bands with apparent molecular weights of 80,000, 68,000, and 60,000 were observed (Fig. 8*B*). Incubation of samples with an increasing amount of unlabeled ligand

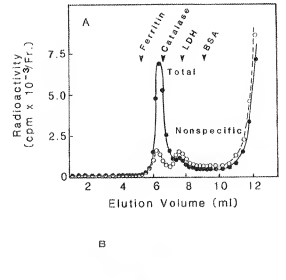

FIG. 8. **Gel filtration (*A*) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (*B*) of affinity-labeled ST receptor.** *A*, membranes were cross-linked with 4 nM ^{125}I-ST in the presence (*O*, nonspecific) or in the absence (●, total) of 1 μM 14-amino acid analog of ST. Samples solubilized with 0.1% Lubrol-PX were subjected to gel filtration as described under "Experimental Procedures." The Stokes radius of cross-linked protein was estimated at 5.5 ± 0.1 nm (mean ± S.E. of three experiments). *B*, autoradiography of affinity-labeled ST receptor on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Cross-linking and solubilization of the membrane were performed as described above. The membrane suspension (*a*, *b*) and the Lubrol-PX solubilized fraction (*c*, *d*) were subjected to electrophoresis using 5–15% gradient gels and to autoradiography as described under "Experimental Procedures." *Lanes b* and *d* show the nonspecific labeling that was observed in the presence of 1 μM 14-amino acid analog of ST. Molecular weight markers were phosphorylase *b* (94,000), bovine serum albumin (67,000) and ovalbumin (43,000). This experiment was repeated three times with similar results.

during cross-linking could not demonstrate an apparent difference in the relative affinities of the three protein bands (Fig. 9). The addition of various protease inhibitors to the buffers used for homogenization and other procedures also did not affect these labeling patterns (data not shown).

DISCUSSION

Dreyfus and Robertson (15) reported the use of CHAPS, a dipolar ionic detergent, for the solubilization of the ST receptor. We found that the yields of solubilized ST receptor and particulate guanylate cyclase with Lubrol-PX was higher than that obtained with CHAPS. Furthermore, sharper separations in various chromatographic procedures were achieved with Lubrol-PX (data not shown). Therefore, in the present study, with an aim being the chromatographic separation of the ST receptor and particulate guanylate cyclase, we used Lubrol-PX as a solubilizing agent. Saturation isotherms of ^{125}I-ST binding to membrane and solubilized preparations were identical (Fig. 1), and displacement patterns of ^{125}I-ST binding to membrane and solubilized preparations by synthetic analogs of ST were also identical (Fig. 2). These results indicate that the toxin-binding site retains its original binding characteristics after its extraction from a lipid-rich membrane environment.

As summarized in Figs. 3 and 4, binding of ^{125}I-ST to membrane and solubilized preparations of receptor was enhanced by various cations. It is noteworthy that similar effects on ligand binding by cations were also observed with specific receptor for atriopeptins (28). To date, ST (7–9) and atriopeptins (29, 30) are the only peptides that specifically activate particulate guanylate cyclase in cell-free systems. The effects of cations on the binding of these heat-stable peptides to their receptors may suggest that the coupling mechanisms of these receptors and particulate guanylate cyclase share some common features. However, this remains to be determined.

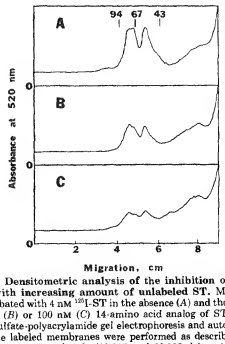

FIG. 9. **Densitometric analysis of the inhibition of the labeling with increasing amount of unlabeled ST.** Membranes were incubated with 4 nM ^{125}I-ST in the absence (*A*) and the presence of 10 nM (*B*) or 100 nM (*C*) 14-amino acid analog of ST. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography of the labeled membranes were performed as described in the legend of Fig. 8. Two bands (80,000 and 68,000 daltons) were not resolved well by the densitometer and appeared as a single broad peak. The labeling of the three bands with ^{125}I-ST was inhibited to a similar extent by unlabeled ST.

With all of the techniques employed the solubilized ST receptor and particulate guanylate cyclase were readily separable (Figs. 5, 6, and 7). Furthermore, the ST receptor migrated as a single peak with all the methods used. These results and the linear Scatchard plots in Fig. 1 indicate that there is a single class of ST receptors and that this macromolecule is separate from particulate guanylate cyclase. We have evidence from studies with adsorption to Con A-agarose and elution with α-methyl mannoside that the solubilized ST receptor is a glycoprotein (data not shown). As reported previously, particulate guanylate cyclase from mammalian tissue (20) and sea urchin sperm (31) is also a glycoprotein. This suggests that both the guanylate cyclase receptor and particulate guanylate cyclase are separate transmembrane glycoproteins that are closely coupled. Although the coupling mechanism between the ST receptor and particulate guanylate cyclase is not presently known, cytoskeletal elements appear to play an important role in this interaction. The evidence in support of this hypothesis is that solubilized preparations fail to increase cyclic GMP synthesis with the addition of ST. However, residual membranes after detergent extraction contains some functionally coupled ST receptor and guanylate cyclase (*i.e.* increased cyclic GMP synthesis with the addition of ST) (15).[2]

When ^{125}I-ST was cross-linked to intestinal brush-border membranes followed by solubilization and analysis on gel filtration chromatography (Fig. 8*A*), the ^{125}I-ST-receptor complex migrated as a sharp single peak similar to the experiments without cross-linking. Mixing experiment with cross-linked and non-cross-linked receptor also exhibited a single peak at the same position (data not shown). These results indicated that the cross-linking procedure did not produce gross alterations in the molecular size of the receptor and that ^{125}I-ST was covalently attached to its receptor and not to some other membrane protein in close proximity. From the gel filtration and sedimentation data the size of the ST receptor is estimated to be about 200,000 daltons. Three peptides (about 80,000, 68,000, and 60,000 daltons) were labeled when cross-linked membrane preparations were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 8*B*). Similar results were obtained when Lubrol-PX-solubilized preparations were used. Incubation of samples with increasing amounts of cold ligand during the cross-linking procedure indicated that these three bands have similar affinities for ST (Fig. 9). These experiments suggest that the ST receptor contains three peptides with binding domains for the toxin. The possibility of receptor heterogeneity with similar affinities cannot be excluded. While similar results were obtained when various protease inhibitors were included in the buffers, we cannot exclude the possibility that these peptides are derived proteolytically from a larger peptide. Previous reports suggesting that binding of ST to its receptor is irreversible presented autoradiographic patterns of ^{125}I-ST-labeled ST receptor using sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the absence of cross-linking (13, 15). In contrast, we observed that the binding of ST to its receptor is reversible and the dissociation of bound ^{125}I-ST can be increased by addition of a large excess of unlabeled ST, as reported by Giannella *et al.* (12). Furthermore, covalent cross-linking of ST to its receptor was required to prevent dissociation of ^{125}I-ST during electrophoresis (data not shown). Although the reasons for these discrepancies are unclear, it is apparent from the present studies that covalent

[2] T. Kuno, Y. Kamisaki, S. A. Waldman, J. Gariepy, G. Schoolnik, and F. Murad, unpublished observations.

affinity labeling of the ST receptor is necessary to study the binding subunit structure of the toxin receptor.

REFERENCES

1. Gyles, C. L. (1971) *Ann. N. Y. Acad. Sci.* **176,** 314–322
2. Banwell, J. G., and Sherr, H. (1973) *Gastroenterology* **65,** 467–497
3. Sack, D. A., Merson, M. H., Wells, J. G., Sack, R. B., and Morris, G. K. (1975) *Lancet* **2,** 239–241
4. Sack, R. B. (1975) *Annu. Rev. Microbiol.* **29,** 333–353
5. Giannella, R. A. (1981) *Annu. Rev. Med.* **32,** 341–357
6. Hughes, J. M., Murad, F., Chang, B., and Guerrant, R. L. (1978) *Nature* **271,** 755–756
7. Field, M., Graf, L. H., Jr., Laird, W. J., and Smith, P. L. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75,** 2800–2804
8. Guerrant, R. L., Hughes, J. M., Chang, B., Robertson, D. C., and Murad, F. (1980) *J. Infect. Dis.* **142,** 220–228
9. Waldman, S. A., O'Hanley, P. D., Falkow, S., Schoolnik, G., and Murad, F. (1984) *J. Infect. Dis.* **149,** 83–89
10. Kimura, H., and Murad, F. (1974) *J. Biol. Chem.* **249,** 6910–6919
11. Quill, H., and Weiser, M. M. (1975) *Gastroenterology* **69,** 470–478
12. Giannella, R. A., Luttrell, M., and Thompson, M. (1983) *Am. J. Physiol.* **245,** 492–498
13. Frantz, J. C., Jaso-Freidman, L., and Robertson, D. C. (1984) *Infect. Immun.* **43,** 622–630
14. Dreyfus, L. A., Jaso-Freidman, L., and Robertson, D. C. (1984) *Infect. Immun.* **44,** 493–501
15. Dreyfus, L. A., and Robertson, D. C. (1984) *Infect. Immun.* **46,** 537–543
16. de Jonge, H. R. (1984) *Biochem. Soc. Trans.* **12,** 180–184
17. Kuno, T., Waldman, S. A., Kamisaki, Y., Gariepy, J., Schoolnik, G., and Murad, F. (1985) *Fed. Proc.* **44,** 1798
18. Alderete, J. F., and Robertson, D. C. (1978) *Infect. Immun.* **19,** 1021–1030
19. Gariepy, J., O'Hanley, P. D., Waldman, S. A., Murad, F., and Schoolnik, G. K. (1984) *J. Exp. Med.* **160,** 1253–1258
20. Waldman, S. A., Lewicki, J. A., Chang, L. Y., and Murad, F. (1983) *Mol. Cell. Biochem.* **57,** 155–166
21. Hauser, H., Howell, K., Dawson, R. M. C., and Bower, D. E. (1980) *Biochim. Biophys. Acta* **602,** 567–577
22. Bruns, R. F., Lawson-Wendling, K., and Pugsley, T. A. (1983) *Anal. Biochem.* **132,** 74–81
23. Steiner, A. L., Parker, C. W., and Kipnis, D. M. (1972) *J. Biol. Chem.* **247,** 1106–1113
24. Hall, J. M., Frankham, P. A., and Strange, P. G. (1983) *J. Neurochem.* **41,** 1526–1532
25. Laemmli, U. K. (1970) *Nature* **227,** 680–685
26. Peterson, G. L. (1977) *Anal. Biochem.* **83,** 346–356
27. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
28. De Lean, A., Gutkowska, J., McNicoll, N., Schiller, P. W., and Cantin, M., and Genest, J. (1984) *Life Sci.* **35,** 2311–2318
29. Waldman, S. A., Rapoport, R. M., and Murad, F. (1984) *J. Biol. Chem.* **259,** 14332–14334
30. Winquist, R. J., Faison, E. P., Waldman, S. A., Schwartz, K., Murad, F., and Rapoport, R. M. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 7661–7664
31. Radany, E. W., Gerzer, R., and Garbers, D. L. (1983) *J. Biol. Chem.* **258,** 8346–8351